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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*
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BELGIUM

Patents ADP number *(if you know it)* 5319390001

If the applicant is a corporate body, give the country/state of its incorporation — **BELGIUM**

4. Title of the invention — **NEUROTROPHIC GROWTH FACTOR**

5. Name of your agent *(if you have one)*
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Signature: Boult Wade Tennant

Date: 14 July 1998

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NEUROTROPHIC GROWTH FACTOR

The present invention is concerned with cloning and expression of a novel member of the GDNF family of neurotrophic factors, designated herein as "enovin" (EVN), and in particular with a nucleic acid sequence encoding enovin, an expression vector comprising said nucleic acid sequence, a host cell transformed with said vector, isolated enovin, compounds which act as agonists or antagonists of enovin and pharmaceutical compositions containing the nucleic acid or the enovin protein or the agonists or antagonists thereof.

Neurotrophic factors are involved in neuronal differentiation, development and maintenance. These proteins can prevent degeneration and promote survival of different types of neuronal cells and are thus potential therapeutic agents for neurodegenerative diseases. Glial cell-line derived neurotrophic factor (GDNF) was the first member of a growing subfamily of neurotrophic factors structurally distinct from the neurotrophins. GDNF is a distantly related member of the transforming growth factor β (TGF-β) superfamily of growth factors, characterized by a specific pattern of seven highly conserved cysteine residues within the amino acid sequence (Kingsley, 1994). GDNF was originally purified using an assay based on its ability to maintain the survival and function of embryonic ventral midbrain dopaminergic neurons *in vitro* (Lin et al., 1993). Other neuronal cell types in the central (CNS) or peripheral nervous systems (PNS) have been shown to be responsive to the survival effects of GDNF (Henderson et al., 1994, Buj-Bello et al., 1995, Mount et al., 1995, Oppenheim et al., 1995). GDNF is produced by cells in an inactive proform, which is cleaved specifically at a RXXR

recognition site to produce active GDNF (Lin et al., 1993). In view of its effects on dopaminergic neurons, clinical trials are evaluating GDNF as a possible treatment for Parkinson's disease, a common neurodegenerative disorder characterized by the loss of a high percentage (up to 70 %) of dopaminergic cells in the substantia nigra of the brain. Exogenous administration of GDNF has potent neuroprotective effects in animal models of Parkinson's disease (Henderson et al., 1994, Beck et al., 1995, Tomac et al., 1995, Yan et al., 1995, Gash et al., 1996, Choi-Lundberg et al., 1997, Bilang-Bleuel et al., 1997, Mandel et al., 1997).

Recently, two new members of the GDNF family of neurotrophic factors have been discovered. Neurturin (NTN) was purified from conditioned medium from Chinese hamster ovary (CHO) cells using an assay based on the ability of growth factors to promote the survival of sympathetic neurons in culture (Kotzbauer et al., 1996). The mature neurturin protein is 57% similar to mature GDNF. Persephin (PSP) was discovered by degenerate primer PCR using genomic DNA. The mature protein, like mature GDNF, promotes the survival of ventral midbrain dopaminergic neurons and of motor neurons in culture (Milbrandt et al., 1998). The similarity of the mature persephin protein with mature GDNF and neurturin is ≈ 50 %.

Both GDNF and NTN require a heterodimeric receptor complex in order to carry out downstream intracellular signal transduction. GDNF binds to the GDNF family receptor alpha 1 (GFRα-1; also termed GDNFRα, RETL1 or TrnR1; GFRα Nomenclature Committee, 1997) subunit, a glycosyl phosphatidyl inositol (GPI)-anchored membrane protein (Jing et al., 1996, Treanor et al., 1996, Sanicola et al., 1997). The GDNF/GFRα-1

complex subsequently binds to and activates the cRET proto-oncogene, a membrane bound tyrosine kinase (Durbec et al., 1996, Trupp et al., 1996), resulting in the phosphorylation of tyrosine residues in cRET and subsequent activation of downstream signal transduction pathways (Worby et al., 1996). Recently GFRα-2 (also termed RETL2, NTNR-α, GDNFR-β or TrnR2), which is similar to GFRα-1, has been identified by a number of different groups (Baloh et al., 1997, Sanicola et al., 1997, Klein et al., 1997, Buj-Bello et al., 1997, Suvanto et al., 1997). The human GFRα-1 and GFRα-2 receptor subunits are 49% identical and 63% similar by protein sequence with 30 of the 31 cysteine residues conserved. Both receptors contain a hydrophobic domain at their carboxy-termini involved in GPI anchoring to the membrane. GFRα-1 and GFRα-2 are widely expressed in almost all tissues and expression may be developmentally regulated (Sanicola et al., 1997, Widenfalk et al., 1997). GFRα-1 is the preferred receptor for GDNF, whereas GFRα-2 preferentially binds neurturin (Jing et al., 1996, Treanor et al., 1996, Klein et al., 1997). It is also clear, however, that there is some cross-talk between these growth factors and receptors as GDNF can bind to GFRα-2 in the presence of cRET (Sanicola et al., 1997) and neurturin can bind to GFRα-1 with low affinity (Klein et al., 1997). GDNF and neurturin are thus part of a neurotrophic signalling system whereby different ligand-binding subunits (GFRα-1 and GFRα-2) can interact with the same tyrosine kinase subunit (cRET). Recently, a third member of the GFRα family of coreceptors, GFRα-3, has been described (Jing et al., 1997, Masure et al., 1998, Worby et al., 1998, Naveilhan et al., 1998, Baloh et al., 1998). The amino acid sequence of GFRα-3 is 35% identical to both GFRα-

1 and GFRα-2. GFRα-3 is not expressed in the developing or adult CNS, but is highly expressed in several developing and adult sensory and sympathetic ganglia of the PNS (Widenfalk et al., 1998, Naveilhan et al., 1998, Baloh et al., 1998). Cells expressing both GFRα-3 and cRET were shown not to respond to either GDNF, NTN or PSP (Worby et al., 1998, Baloh et al., 1998). Novel members of the GDNF family of neurotrophic factors and their receptors may be discovered in the future, as persephin is an orphan ligand while GFRα-3 is an orphan receptor.

In the present application, there is described a novel neurotrophic factor sequence belonging to the GDNF family.

Therefore according to a first aspect of the present invention there is provided a nucleic acid molecule encoding a human neurotrophic growth factor designated herein as enovin and having the amino acid sequence illustrated in Figure 1, or encoding a functional equivalent, derivative or bioprecursor of said growth factor, which nucleic acid is preferably DNA and even more preferably a cDNA molecule.

Advantageously, the nucleic acid according to the invention may be used to express the human neurotrophic growth factor according to the invention, in a host cell or the like using an appropriate expression vector. Preferably, the nucleic acid according to the invention comprises the sequence from positions 80 to 418 of the sequence illustrated in Figure 1 and even more preferably the complete sequence illustrated in Figure 1. There is also provided by the invention a nucleic acid sequence capable of hybridising to any of the nucleic acid sequences according to the invention, under high stringency conditions, which would be well known to

those skilled in the art.

Stringency of hybridisation as used herein refers to conditions under which polynucleic acids are stable. The stability of hybrids is reflected in the melting temperature (Tm) of the hybrids. Tm can be approximated by the formula:

$$81.5°C-16.6(10g10[Na^+]+0.41\ (\%G\&C)-6000/e$$

wherein l is the length of the hybrids in nucleotides. Tm decreases approximately by 1-15°C with every 1% decrease in sequence homology.

An expression vector according to the invention includes vectors capable of expressing DNA operatively linked to regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector may include a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector may include a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well known in the art.

Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that upon introduction into an appropriate host cell results in

expression of the DNA or RNA fragments. Appropriate expression vectors are well known to those skilled in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

The nucleic acid sequence capable of hybridising to the nucleic acid according to the invention may comprise, for example, antisense DNA which may either be used as a probe or as a medicament or in a pharmaceutical composition.

A further aspect of the invention comprises the host cell transformed, transfected or infected with the expression vector according to the invention, which cell preferably comprises a eukaryotic cell and more preferably a mammalian cell.

Incorporation of cloned DNA into a suitable expression vector for subsequent transformation of said cell and subsequent selection of the transformed cells is well known to those skilled in the art as provided in Sambrook et al (1989) Molecular Cloning, A Laboratory manual, Cold Spring Harbour Laboratory Press.

A further aspect of the present invention comprises a nucleic acid sequence of at least 15 nucleotides of the nucleic acid sequences according to the invention and preferably from 15 to 15 nucleotides.

These sequences may, advantageously be used as probes or primers to initiate replication or the like. Such nucleic acid sequences may be produced according to techniques well known in the art, such as by recombinant or synthetic means. They may also be used in diagnostic kits or devices or the like for detecting for the presence of a nucleic acid according

to the invention. These tests generally comprise contacting the probe with a sample under hybridising conditions and detecting for the presence of any duplex formation between the probe and any nucleic acid in the sample. Nucleic acid sequences according to the invention may also be produced using recombinant or synthetic means such as described in Sambrook et al (Molecular Cloning: A Laboratory Manual,1989). Advantageously, human allelic variants or polymorphisms of the DNA sequence according to the invention may be identified by, for example, probing cDNA or genomic libraries from a range of individuals for example from different populations. Furthermore, nucleic acids and probes according to the invention may be used to sequence genomic DNA from patients using techniques well known in the art, such as the Sanger Dideoxy chain termination method, which may advantageously ascertain any predisposition of a patient to certain disorders associated with a growth factor according to the invention.

Further provided by the present invention is a transgenic cell, tissue or organism comprising a transgene capable of expressing the human neurotrophic factor enovin according to the invention.

The term "transgene capable of expression" as used herein means any suitable nucleic acid sequence which leads to expression of a neurotrophic factor having the same function or activity as a neurotrophic factor according to the invention. The transgene may include, for example, genomic nucleic acid isolated from human cells or synthetic nucleic acid including cDNA, integrated into the chromosome or in an extrachromosomal state.

Preferably, the transgene comprises a vector according to the invention, which vector includes a

nucleic acid sequence encoding said neurotrophic factor, or a functional fragment of said nucleic acid. A "functional fragment" of said nucleic acid should be taken to mean a fragment of the gene or cDNA encoding said neurotrophic factor or a functional equivalent thereof, which fragment is capable of being expressed to produce a functional growth factor according to the invention. Thus, for example, fragments of the neurotrophic factor according to the invention which correspond to the specific amino acid residues interacting with the corresponding receptor also form part of the present invention and which fragments may serve to function as agonists activating the growth factors receptor to elicit its growth promoting and survival sustaining effects on cells. This aspect of the invention also includes differentially spliced isoforms and transcriptional starts of the nucleic acids according to the invention.

In accordance with the present invention, a defined nucleic acid includes not only the identical nucleic acid but also any minor base variations including in particular, substitutions in bases which result in a synonymous codon (a different codon specifying the same amino acid residue) due to the degenerate code in conservative amino acid substitutions. The term "nucleic acid sequence" also includes the complementary sequence to any single stranded sequence given regarding base variations.

Furthermore, a defined protein, polypeptide or amino acid sequence according to the invention includes not only the identical amino acid sequence but isomers thereof in addition to minor amino acid variations from the natural amino acid sequence including conservative amino acid replacements (a replacement by an amino acid that is related in its

side chains). Also included are amino acid sequences which vary from the natural amino acid but result in a polypeptide which is immunologically identical or similar to the polypeptide encoded by the naturally occurring sequence.

Neurotrophic growth factors expressed by the host cells according to the invention are also encompassed within the present invention.

Because of the sequence similarity between the growth factor described herein with previously identified growth factors of the GDNF family enovin is also believed to be capable of promoting cell survival and growth and in treating disorders resulting from defects in function or expression of said neurotrophic factor.

The nucleic acid molecules or the neurotrophic factor according to the invention may, advantageously, therefore be used as a medicament or alternatively in the preparation of a medicament to promote maintenance and survival of neuronal cells and for treating neuronal disorders including Parkinson's disease, Alzheimer's disease, peripheral neuropathy, amyotrophic lateral sclerosis, peripheral nerve trauma or injury and exposure to neurotoxins.

The nucleic acids and neurotrophic factor according to the invention may also be included in a pharmaceutical composition together with a pharmaceutically acceptable carrier, diluent or excipient therefor.

Antibodies to the neurotrophic factor of the present invention may, advantageously, be prepared by techniques which are known in the art. For example, polyclonal antibodies may be prepared by inoculating a host animal such as a mouse with the growth factor or an epitope thereof and recovering immune serum.

Monoclonal antibodies may be prepared according to known techniques such as described by Kohler R. and Milstein C., Nature (1975) 256, 495-497.

Antibodies according to the invention may, advantageously, be used in a method of detecting for the presence of a growth factor according to the invention, which method comprises reacting the antibody with a sample and identifying any protein bound to said antibody. A kit is also provided for performing said method which comprises an antibody according to the invention and means for reacting the antibody with said sample.

Proteins which interact with the neurotrophic factor of the invention, such as for example it's corresponding cellular receptor may be identified by investigating protein-protein interactions using the two-hybrid vector system first proposed by Chien *et al* (1991).

This technique is based on functional reconstitution in vivo of a transcription factor which activates a reporter gene. More particularly the technique comprises providing an appropriate host cell with a DNA construct comprising a reporter gene under the control of a promoter regulated by a transcription factor having a DNA binding domain and an activating domain, expressing in the host cell a first hybrid DNA sequence encoding a first fusion of a fragment or all of a nucleic acid sequence according to the invention and either said DNA binding domain or said activating domain of the transcription factor, expressing in the host at least one second hybrid DNA sequence, such as a library or the like, encoding putative binding proteins to be investigated together with the DNA binding or activating domain of the transcription factor which is not incorporated in the first fusion;

detecting any binding of the proteins to be investigated with a protein according to the invention by detecting for the presence of any reporter gene product in the host cell; optionally isolating second hybrid DNA sequences encoding the binding protein.

An example of such a technique utilises the GAL4 protein in yeast. GAL4 is a transcriptional activator of galactose metabolism in yeast and has a separate domain for binding to activators upstream of the galactose metabolising genes as well as a protein binding domain. Nucleotide vectors may be constructed, one of which comprises the nucleotide residues encoding the DNA binding domain of GAL4. These binding domain residues may be fused to a known protein encoding sequence, such as for example the nucleic acids according to the invention. The other vector comprises the residues encoding the protein binding domain of GAL4. These residues are fused to residues encoding a test protein, preferably from the signal transduction pathway of the vertebrate in question. Any interaction between neurotrophic factor encoded by the nucleic acid according to the invention and the protein to be tested leads to transcriptional activation of a reporter molecule in a GAL-4 transcription deficient yeast cell into which the vectors have been transformed. Preferably, a reporter molecule such as ß-galactosidase is activated upon restoration of transcription of the yeast galactose metabolism genes.

Once the receptor has been identified for the growth factor according to the invention assays may be prepared to identify agonist or antagonistic compounds of the growth factor which compounds may themselves be used as medicaments or in the preparation of a medicament to promote maintenance and survival of

neuronal cells and for treating neuronal disorders including Parkinson's disease, Alzheimer's disease, peripheral neuropathy, amyotrophic lateral sclerosis, peripheral nerve trauma or injury and exposure to neurotoxins. Such compounds may also be included in pharmaceutical compositions together with a pharmaceutically acceptable carrier, diluent or excipient therefor.

The present invention may be more clearly understood by the following examples which are purely exemplary and by reference to the accompanying drawings wherein:

Figure 1: is partial cDNA sequence of a neurotrophic factor according to the invention designated as enovin. The consensus sequence was obtained by PCR amplification with primers PNHsp3 and PNHap1 on different cDNAs and on genomic DNA followed by cloning and sequence analysis and comparison of the obtained sequences. The predicted one letter code amino acid sequence is shown above the DNA sequence. The nucleotide residue number is shown on the right of the DNA sequence, whereas the amino acid residue number is shown to the right of the translated protein sequence. The putative RXXR cleavage site for the prodomain is indicated in bold and underlined. The putative start of the mature protein is indicated by an arrow. The seven conserved cysteine residues characteristics for all members of the TGF-β family are indicated in bold. A potential N-glycosylation site is double underlined,

Figure 2: is alignment of the predicted mature protein sequences of human GDNF, NTN, PSP and EVN. The sequences were aligned using the ClustalW

alignment program. Amino acid residues conserved between all three proteins are included in the black areas. Residues conserved between two or three of the sequences are shaded in grey. The 7 conserved cysteine residues characteristic for members of the TGF-β family are indicated by asterisks above the sequence. Amino acid residues are numbered to the right. The dashes indicate gaps introduced into the sequence to optimize the alignment,

Figure 3: is partial cDNA sequence of enovin. The consensus sequence was obtained by PCR amplification (primary PCR with primers PNHsp1 and nested PCR with primers PNHsp2 and PNHap2) on different cDNAs followed by cloning and sequence analysis and comparison of the obtained sequences. The translated one letter code amino acid sequence of nucleotides 30 to 284 (reading frame A) is shown above the sequence and numbered to the right (A1 to A85). This reading frame contains a putative ATG translation start codon. The translated one letter code amino acid sequence of nucleotides 334 to 810 (reading frame B) is shown above the sequence and numbered to the right (B1 to B159). This reading frame contains the region of homology with GDNF, NTN and PSP. The nucleotide residue number is shown to the right of the DNA sequence. The putative RXXR cleavage site for the prodomain is indicated in bold and underlined. The putative start of the mature protein is indicated by an arrow. The seven conserved cysteine residues characteristic for all members of the TGF-β family are indicated in bold. A potential N-glycosylation site is double underlined.

Materials and methods.

Materials.

Native Taq polymerase, ampicillin, IPTG (isopropyl-β-D-thiogalactoside), X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and all restriction enzymes used were from Boehringer Mannheim (Mannheim, Germany). 10 mM dNTP mix was purchased from Life Technologies (Gaithersburg, MD, USA). The TOPO-TA cloning kit was purchased from Invitrogen BV (Leek, The Netherlands). The Qiagen plasmid mini- or midi-DNA purification kit, the Qiaprep Spin Miniprep kit and the Qiaquick gel extraction kit were purchased from Qiagen GmbH (Düsseldorf, Germany). cDNA libraries, Marathon™ Ready cDNA kits, human multiple tissue cDNA (MTC™) panels I and II and the Advantage-GC cDNA PCR kit were obtained from Clontech Laboratories (Palo Alto, CA, USA). All PCR reactions were performed in a GeneAmp PCR system 9600 cycler (Perkin Elmer, Foster City, CA, USA). LB (Luria-Bertani) medium consists of 10 g/l of tryptone, 5 g/l of yeast extract and 10 g/l of NaCl. 2x YT/ampicillin plates consist of 16 g/l of tryptone, 10 g/l of yeast extract, 5 g/l of NaCl, 15 g/l of agar and 100 mg/l of ampicillin.

Database homology searching and sequence comparison.

Using the complete human glial cell-line derived neurotrophic factor (GDNF; accession no. Q99748), neurturin (NTN; accession no. P39905) and persephin (PSP; accession no. AF040962) cDNA derived protein sequences as query sequences, a BLAST (Basic Local Alignment Search Tool; Altschul et al., 1990) search

was performed on the daily update of the EMBL/GenBank human expressed sequence tag (EST) and genomic databases.

Additional BLAST searches were performed using the genomic sequence with accession no. AC005038 and several ESTs present in the GenBank database and showing homology to this genomic sequence were detected.

The percentage identity and percentage similarity between members of the GDNF family was calculated by pairwise comparison of the sequences using the BESTFIT program (Genetics Computer Group sequence analysis software package, version 8.0, University of Wisconsin, Madison, WI, USA). Alignments of DNA or protein sequences were done with the ClustalW alignment program (EMBL, Heidelberg, Germany).

Oligonucleotide synthesis for PCR and DNA sequencing.

All oligonucleotide primers were ordered from Eurogentec (Seraing, Belgium). Insert-specific sequencing primers (15- and 16-mers) and primers for use in PCR reactions were designed manually. DNA was prepared on Qiagen-tip-20 or -100 anion exchange or Qiaquick spin columns (Qiagen GmbH, Düsseldorf, Germany) and recovered from the columns in 30 μl TE-buffer (10 mM Tris.HCl, 1 mM EDTA (sodium salt), pH 8.0).

Sequencing reactions were done on both strands using the ABI prism BigDye Terminator Cycle sequencing kit and were run on an Applied Biosystems 377XL sequencer (Perkin Elmer, ABI Division, Foster City, CA, USA). The Sequencher™ software was used for sequence assembly and manual editing (GeneCodes,

AnnArbor, MI, USA).

Cloning of a novel GDNF homologue.

A DNA region spanning nucleotides 67411 to 68343 of EMBL accession no. AC005038 of which the translated protein sequence was homologous to mature NTN and PSP was used to design oligonucleotide primers for PCR amplification. The different primers used are shown in Table 1.

Table 1: Primers used for the PCR amplification of fragments of AC005038.

Primer name	Primer sequence
PNHsp1	5' - CGGTGCACTCAGGTGATTCCTCC - 3'
PNHsp2	5' - GGCAGCAAACCCATTATACTGGAACC - 3'
PNHsp3	5' - CGCTGGTGCAGTGGAAGAGCC - 3'
PNHsp4	5' - CTGCACCCCCATCTGCTCTTCC - 3'
PNHap1	5' - GCAGGAAGAGCCACCGGTAAGG - 3'
PNHap2	5' - CCAGTCTGCAAAGCCCTGGAGC - 3'

Primers PNHsp3 and PNHap1 were used to amplify a fragment of 495 bp on cDNA derived from different human tissues (fetal brain, whole fetus, prostate or lung Marathon-Ready™ cDNA (Clontech Laboratories), frontal cortex, hippocampus and cerebellum cDNA) and on human genomic DNA. Based on the genomic sequence from the EMBL/GenBank database (acc. no. AC005038), the fragment to be amplified was predicted to have a G+C content of 76 %. Therefore, amplifications were done using the Advantage-GC cDNA PCR kit (Clontech Laboratories, Palo Alto, CA, USA) optimized for the amplification of GC-rich DNA sequences. PCR reactions were performed in a total volume of 50 μl, containing 1x GC cDNA PCR reaction buffer, 0.2 mM dNTP, 1 M GC-MELT™, 200 nM of primers PNHsp3 and PNHap1, 1 μl of Advantage KlenTaq polymerase mix and 1 to 5 μl of cDNA

or 0.5 μg of genomic DNA. Samples were heated to 95°C for 5 min and cycling was done for 45 s at 95°C, 1 min at 58°C and 40 s at 72°C for 35 cycles, with a final step of 7 min at 72°C. Samples were finally treated with 2.5 U of native Taq DNA polymerase to add an A-overhang. PCR products were analysed on a 1% (w/v) agarose gel in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA (sodium salt), pH 8.3). PCR fragments of the expected size (495 bp) were excised from the gel and purified with the Qiaquick gel extraction kit. The PCR fragments were sequenced to confirm their identity and cloned into the plasmid vector pCR2.1-TOPO using the TOPO TA cloning kit according to manufacturer's instructions. Approximately 20 ng of purified fragment was combined with 1 μl pCR2.1-TOPO vector in a total volume of 5 μl. Reactions were incubated at room temperature (20°C) for 5 min. 2 μl of the reaction was transformed into TOP10F' competent cells (Invitrogen BV) using heat-shock transformation and plated on 2x YT/ampicillin plates supplemented with 10 mM IPTG and 2% (w/v) X-gal for blue-white screening. White colonies after overnight growth were picked from the plates, grown in 5 ml of LB medium supplemented with 100 mg/l ampicillin and plasmid DNA prepared using the Qiaprep Spin Miniprep kit. The presence of an insert of the expected size was confirmed by restriction digestion with *Eco*RI. The plasmid insert of several positive clones was sequenced and the obtained sequences compared using the ClustalW alignment program.

To obtain additional coding sequence for the novel GDNF homologue, a fragment with an expected size of 931 bp based on the EMBL/GenBank sequence (acc. no. AC005038) was amplified by PCR using primers PNHsp1 and PNHap1. PCR reactions were performed in a total

volume of 50 µl, containing 1x GC cDNA PCR reaction buffer, 0.2 mM dNTP, 1 M GC-MELT™, 200 nM of primers PNHsp1 and PNHap1, 1 µl of Advantage KlenTaq polymerase mix and 1 to 5 µl of cDNA from cerebellum, frontal cortex or hippocampus or 0.5 µg of genomic DNA. Samples were heated to 95°C for 5 min and cycling was done for 45 s at 95°C, 1 min at 58°C and 1 min 30 s at 72°C for 35 cycles, with a final step of 7 min at 72°C. PCR products were analysed on a 1% (w/v) agarose gel in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA (sodium salt), pH 8.3). A second round amplification was performed with nested primers (PNHsp2 and PNHap2). 1 µl of the first round amplification reaction was used in a total volume of 50 µl, containing 1x GC cDNA PCR reaction buffer, 0.2 mM dNTP, 1 M GC-MELT™, 200 nM of primers PNHsp2 and PNHap2 and 1 µl of Advantage KlenTaq polymerase mix. Samples were heated to 95°C for 5 min and cycling was done for 45 s at 95°C, 1 min at 58°C and 1 min 30 s at 72°C for 35 cycles, with a final step of 7 min at 72°C. Samples were analysed on a 1% (w/v) agarose gel in 1x TAE buffer. PCR fragments of the expected size (870 bp) were excised from the gel and purified with the Qiaquick gel extraction kit. The PCR fragments were sequenced to confirm their identity, treated with 2.5 U of Taq polymerase and cloned into the plasmid vector pCR2.1-TOPO using the TOPO TA cloning kit according to manufacturer's instructions. Approximately 20 ng of purified fragment was combined with 1 µl pCR2.1-TOPO vector in a total volume of 5 µl. Reactions were incubated at room temperature (20°C) for 5 min. 2 µl of the reactions was transformed into TOP10F' competent cells using heat-shock transformation and plated on 2x YT/ampicillin plates supplemented with 10 mM IPTG and 2% (w/v) X-gal for blue-white screening. White

colonies after overnight growth were picked from the plates, grown in 5 ml of LB medium supplemented with 100 mg/l ampicillin and plasmid DNA prepared using the Qiaprep Spin Miniprep kit. The presence of an insert of the expected size was confirmed by restriction digestion with *Eco*RI. The plasmid insert of several positive clones was sequenced and the sequences compared using the ClustalW alignment program.

Analysis of enovin gene expression by RT-PCR analysis.

Oligonucleotide primers PNHsp3 and PNHap1 (see table 1) were used for the specific PCR amplification of a 502 bp fragment from enovin. PCR amplifications were performed on human multiple tissue cDNA (MTC™) panels normalised to the mRNA expression levels of six different housekeeping genes. PCR reactions with enovin specific primers were performed in a total volume of 50 μl, containing 5 μl of cDNA, 1x GC cDNA PCR reaction buffer, 0.2 mM dNTP, 1 M GC-MELT™, 400 nM of primers PNHsp3 and PNHap1 and 1 μl of Advantage KlenTaq polymerase mix. Samples were heated to 95°C for 30 s and cycling was done for 30 s at 95°C and 30 s at 68°C for 35 cycles. Samples were analysed on a 1.2 % (w/v) agarose gel in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA (sodium salt), pH 8.3) and images of the ethidium bromide stained gels were obtained using an Eagle Eye II Video system (Stratagene, La Jolla, CA, USA).

Similarity searching of the daily update of the EMBL/GenBank database with the human neurturin and persephin protein sequences yielded a genomic DNA sequence coding for a putative novel protein similar to the neurotrophic factors GDNF, NTN and PSP which

has been called enovin (EVN). Additional database homology searching using the genomic DNA sequence surrounding the region coding for enovin yielded several expressed sequence tag (EST) clones derived from different human tissues (prostate epithelium [accession no. HS1322952], lung carcinoma [accession no. AA931637] and parathyroid tumor [accession no. AA844072]). These clones contain DNA sequence outside of the region of homology with GDNF, PSP or NTN, but confirmed that enovin mRNA is expressed in normal and tumor tissues.

Initial PCR amplification using primers (PNHsp3 and PNHap1) based on the genomic sequence yielded a fragment of ≈500 bp from fetus, fetal brain, prostate, frontal cortex, hippocampus, cerebellum cDNA and from genomic DNA, but not from lung cDNA. Cloning and sequence analysis of these fragments yielded a DNA sequence of 474 bp that translated into a predicted protein sequence of 139 amino acid residues including seven conserved cysteine residues characteristic of all the members of the transforming growth factor β (TGF-β) family of proteins (Kingsley, 1994) (Figure 1). The sequence also contained a RXXR motif for cleavage of the prodomain (RAAR, amino acid position 23 to 26) (Barr, 1991). A similar cleavage site is present in the GDNF, NTN and PSP protein sequences, at a comparable position in the prodomain sequence. Assuming cleavage of the enovin prodomain occurs at this site *in vivo*, the mature EVN protein sequence contains 113 amino acid residues (residue 27 to 139 in figure 1) and has a calculated molecular mass of 11965 Da and an isoelectric point of 11.8. There is one potential N-glycosylation site present in the mature sequence (NST at amino acid position 121-123). Moreover, several regions conserved between the mature

forms of the known neurotrophic factors GDNF, NTN and PSP were also present in enovin (Figure 2). Table 2 summarizes the results of the comparison of the mature protein sequences of the GDNF family members by the BESTFIT program. Percentage identity and percentage similarity are shown. The GDNF, NTN, PSP and EVN mature sequences used in this comparison started at the first amino acid residue following the RXXR cleavage site.

Table 2: Pairwise comparison of mature human GDNF family members using the BESTFIT program.

Comparison	% identity	% similarity
EVN vs GDNF	38.8	47.2
EVN vs NTN	51.0	56.1
EVN vs PSP	53.3	57.8
GDNF vs NTN	44.8	57.3
GDNF vs PSP	44.3	50.0
NTN vs PSP	50.0	54.4

From these comparisons it is apparent that the mature enovin protein is more closely related to persephin and to neurturin than to GDNF.

Amplification, cloning and sequence analysis of a larger fragment of the enovin DNA sequence from frontal cortex cDNA using primers based on the genomic EMBL/GenBank sequence (acc. no. AC005038) yielded a sequence of 819 bp (Figure 3). This sequence contains a putative ATG start codon at nucleotide positions 30-32 and yields an open reading frame (reading frame A in figure 3) that extends up to a stop codon at nucleotide positions 285-287. The translated protein sequence of this region does not show similarity to any known protein in the databases. Translation of the cDNA sequence in the second reading frame (reading frame B in figure 3) yields a predicted protein sequence of 159 amino acid residues. This sequence

contains the RXXR cleavage site (position B43 to B46; nucleotide position 460-471) and the sequence corresponding to the mature enovin sequence (position B47 to B159; nucleotide position 472-810). The open reading frame including the RXXR cleavage site and the mature enovin coding sequence extends from nucleotide position 334 (preceded by an in-frame stop codon) to a stop codon at position 811-813, but does not contain an ATG codon for a starting methionine residue. In analogy with persephin (Milbrandt et al., 1998) we hypothesize that an unspliced intron is present in the majority of the mRNA transcripts from the EVN gene. GDNF and NTN also have an intron in their respective prodomain coding regions (Matsushita et al., 1997, Heuckeroth et al., 1997).

Analysis of enovin gene expression on mRNA derived from different human tissues by RT-PCR analysis shows that enovin mRNA is expressed in all tissues tested, including brain, heart, kidney, liver, lung, pancreas, skeletal muscle, colon, small intestine, peripheral blood leukocytes, spleen, thymus, prostate, testis, ovary and placenta. Although the expression of NTN and PSP in different tissues has not been fully characterized, their expression levels seem much lower and the expression more restricted to certain tissues (Kotzbauer et al., 1996, Milbrandt et al., 1998).

Since GDNF, NTN and PSP all promote the maintenance and survival of different types of neuronal cells, it is anticipated that enovin has similar biological effects on nerve cells and, possibly, on other cell types too. Therefore, it could be envisaged that the enovin protein might be useful in the treatment of neural disorders in general, including Parkinson's disease, Alzheimer's disease,

peripheral neuropathy, amyotrophic lateral sclerosis (ALS), Huntington's disease, acute brain injury, nervous system tumors, multiple sclerosis, peripheral nerve trauma or injury and exposure to neurotoxins. Both GDNF and NTN have been shown to signal via a signalling complex composed of a ligand-binding subunit, either GFRα-1 or GFRα-2, and a signalling subunit, the cRET protein tyrosine kinase. Enovin is expected to exert its biological effects via a similar signalling complex composed of a GFRα binding partner (either GFRα-1, GFRα-2, the recently characterised orphan receptor GFRα-3 or other as yet uncharacterized members of the GFRα family) in combination with cRET or another signalling partner.

In humans, germ line mutations in GDNF or cRET can lead to several disease phenotypes including multiple endocrine neoplasia and Familial Hirschsprung disease (HSCR) (Romeo et al., 1994, Edery et al., 1994, Angrist et al., 1996). Both diseases are associated with gut dismotility, with Hirschsprung disease being the most common cause of congenital bowel obstruction in infants. Interestingly, GDNF and cRET knockout mice exhibit remarkably similar pathologies with renal agenesis and intestinal aganglionosis (Sanchez et al., 1996; Moore et al., 1996; Pichel et al., 1996). Enovin could be involved in similar disorders of the gut or the kidneys or, since it is ubiquitously expressed, could be important in the development of other peripheral organs in the body.

The interaction of ligands with their receptors is generally achieved by the interaction of specific bonds from particular residues in both proteins. Fragments of a protein can serve as agonists activating the receptor to elicit its growth promoting

and survival sustaining effects on cells. Parts of enovin or synthetic peptides based on the enovin protein sequence can therefore be useful as agonists or antagonists to regulate the receptor. Using peptide synthesis or recombinant techniques, hybrid growth factors composed of parts of GDNF, NTN or PSP or any other neurotrophic or growth factor with parts of enovin can be produced to yield a novel synthetic growth factor with new properties.

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List of abbreviations.

BLAST	basic local alignment search tool
bp	base pairs
cDNA	complementary DNA
CNS	central nervous system
EST	expressed sequence tag
EVN	enovin
GDNF	glial cell-line derived neurotrophic factor
GFRα	GDNF family receptor α
GPI	glycosyl phosphatidyl inositol
MTC	multiple tissue cDNA
NTN	neurturin
PCR	polymerase chain reaction
PNS	peripheral nervous system
PSP	persephin
RT-PCR	reverse transcription PCR
TGF-β	transforming growth factor β

Claims

1. A nucleic acid molecule encoding a human neurotrophic growth factor designated enovin and having the amino acid sequence illustrated in Figure 1, or encoding a functional equivalent, derivative or bioprecursor of said growth factor.

2. A nucleic acid molecule according to claim 1 which is a DNA molecule.

3. A nucleic acid molecule according to claim 1 or 2 which is a cDNA molecule.

4. A nucleic acid molecule according to any preceding claim having the nucleic acid sequence from positions 80 to 418 illustrated in Figure 1.

5. A nucleic acid according to any preceding claim having the nucleic acid sequence illustrated in Figure 1.

6. A nucleic acid molecule capable of hybridising to the sequence of any of claims 1 to 5 under high stringency conditions.

7. A human neurotrophic growth factor encoded by the nucleic acid molecule according to any of claims 1 to 5.

8. A growth factor according to claim 6 having the amino acid sequence from position 27 to 139 of the amino acid sequence illustrated in Figure 1.

9. A growth factor according to claim 7 or 8

having the amino acid sequence illustrated in Figure 1 or a functional equivalent, derivative or bioprecursor of said growth factor.

10. A expression vector comprising a DNA molecule according to any of claims 2 to 5.

11. An expression vector according to claim 10 which includes a sequence encoding a reporter molecule.

12. A host cell transformed or transfected with the vector according to claims 10 or 11.

13. A host cell according to claim 12 which cell is a eukaryotic cell.

14. A transgenic cell, tissue or organism comprising a transgene capable of expressing human neurotrophic factor enovin according to any of claims 7 to 9.

15. A transgenic cell, tissue or organism according to claim 14, wherein said transgene comprises a vector according to claims 10 or 11.

16. A neurotrophic growth factor or a functional equivalent, derivative or bioprecursor thereof, expressed by a cell according to any of claims 12 to 15.

17. A nucleic acid molecule according to any of claims 1 to 6 for use as a medicament.

18. Use of a nucleic acid molecule according to

any of claims 1 to 6 in the manufacture of a medicament to promote maintenance and survival of neuronal cells and for treating neural disorders including Parkinson's disease, Alzheimer's disease, Huntingdons disease, peripheral neuropathy, acute brain injury, nervous system tumours, multiple sclerosis, amyotrophic lateral sclerosis, peripheral nerve trauma or injury exposure to neurotoxins, multiple endocrine neoplasia and familial Hirschsprung disease.

19. A growth factor according to any of claims 7 to 9 for use as a medicament.

20. Use of a growth factor according to any of claims 7 to 9 in the manufacture of a medicament to promote maintenance and survival of neuronal cells and for treating neural disorders including Parkinson's disease, Alzheimer's disease, Huntingdon's disease, peripheral neuropathy, acute brain injury, nervous system tumours, multiple sclerosis, amyotrophic lateral sclerosis, peripheral nerve trauma or injury exposure to neurotoxins, multiple endocrine neoplasia and familial Hirschsprung disease.

21. A pharmaceutical composition comprising a nucleic acid molecule according to any of claims 1 to 6 or growth factor according to any of claims 7 to 9 together with a pharmaceutically acceptable carrier, diluent or excipient therefor.

22. An antibody capable of binding to a growth factor according to any of claims 7 to 9 or an epitope thereof.

23. A method of detecting for the presence of a growth factor according to any of claims 7 to 9 in a sample which method comprises reacting an antibody according to claim 22 with said sample and detecting for binding of said antibodies with said growth factor.

24. A method according to claim 23 wherein said antibody is conjugated to a reporter molecule.

25. A kit or device for detecting for the presence of a growth factor in a sample comprising an antibody according to claim 23 and means for reacting said antibody and said sample.

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  P  P  Q  P  S  R  P  A  P  P  P  P  A  P  P  S      16
cgccgccgcagccttctcggcccgcgcccccgccgcctgcacccccatct    50

 A  L  P  R  G  G  R  A  A  R  A  G  G  P  G  S  R    33
gctcttccccgcgggggccgcgcggcgcgggctgggggcccgggcagccg    100

  A  R  A  A  G  A  R  G  C  R  L  R  S  Q  L  V     49
cgctcgggcagcgggggcgcggggctgccgcctgcgctcgcagctggtgc    150

 P  V  R  A  L  G  L  G  H  R  S  D  E  L  V  R  F    66
cggtgcgcgcgctcggcctgggccaccgctccgacgagctggtgcgtttc    200

 R  F  C  S  G  S  C  R  R  A  R  S  P  H  D  L  S    83
cgcttctgcagcggctcctgccgccgcgcgcgctctccacacgacctcag    250

  L  A  S  L  L  G  A  G  A  L  R  P  P  P  G  S     99
cctggccagcctactgggcgccggggccctgcgaccgccccgggctccc    300

 R  P  V  S  Q  P  C  C  R  P  T  R  Y  E  A  V  S    116
ggcccgtcagccagccctgctgccgacccacgcgctacgaagcggtctcc    350

 F  M  D  V  N  S  T  W  R  T  V  D  R  L  S  A  T    133
ttcatggacgtcaacagcacctggagaaccgtggaccgcctctccgccac    400

                                                       139
  A  C  G  C  L  G  *
cgcctgcggctgcctgggctgagggctcgctccagggctttgcagactgg    450

                                                       474
accettaccggtggctcttcctgc
```

Figure 1

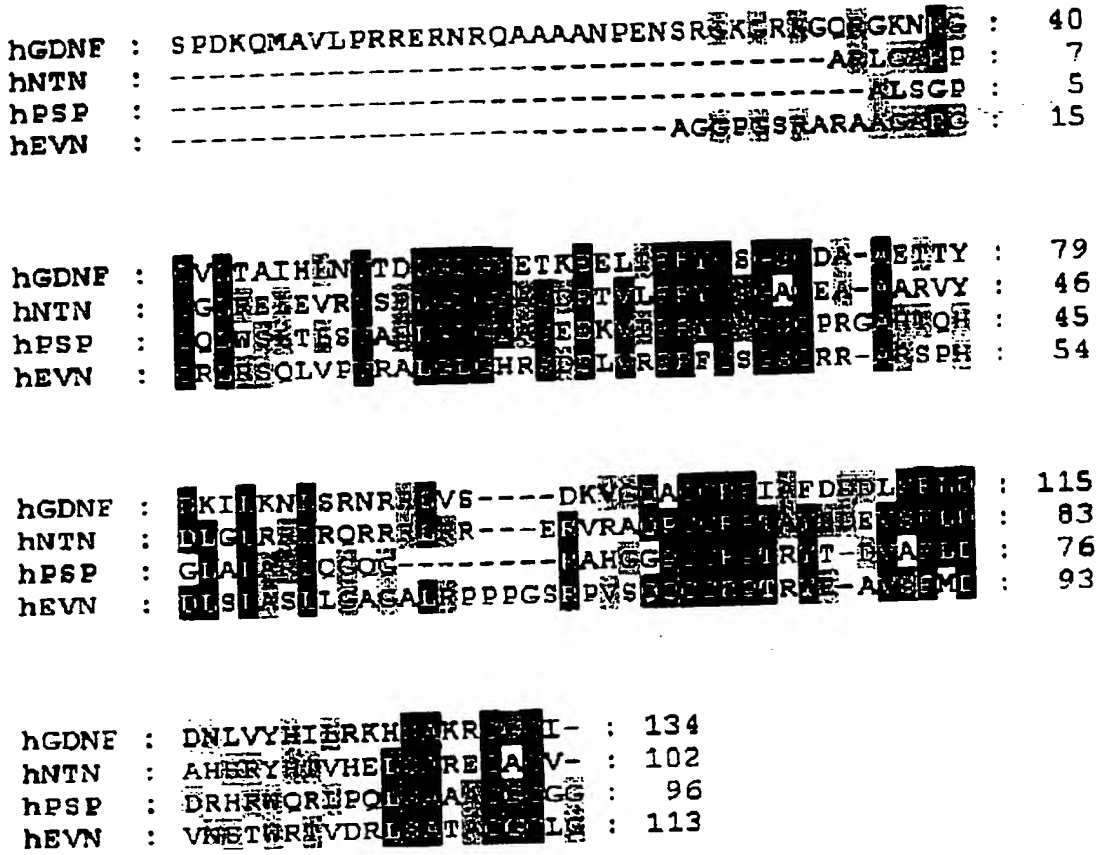

Figure 2

reading frame A M P G L I S A 7
gagtttcccctccacacagctaggagcccatgcccggcctgatctcagcc 50

R G Q P L L E V L P P Q A H L G A 24
cgaggacagcccctccttgaggtccttcctccccaagcccacctgggtgc 100

L F L P E A P L G L S A Q P A L A40
cctctttctccctgaggctccacttggtctctccgcgcagcctgccctgt 150

W P T L A A L A L L S S V A E A S A57
ggcccaccctggccgctctggctctgctgagcagcgtcgcagaggcctcc 200

L G S A P R S P A P R E G P P P V A74
ctgggctccgcgccccgcagccctgccccccgcgaaggcccccgcctgt 250

L A S P A G H L P G R * A85
cctggcgtcccccgccggccacctgccgggtaggtgagagggcgagggggg 300

reading frame B * L G L I P G B6
cggggcggggctggcccgggacaccgcgcgtgactgggtctcattccagg 350

G R T A R W C S G R A R R P P P B22
gggacgcacggcccgctggtgcagtggaagagcccggcggccgccgccgc 400

Q P S R P A P P P P P A P P S A L P B39
agccttctcggcccgcgcccccgccgcctgcacccccatctgctcttccc 450

R G G **R A A R** A G G P G S R A R A B56
cgcgggggccgcgcggcgcgggctgggggcccgggcagccgcgctcgggc 500

A G A R G **C** R L R S Q L V P V R B72
agcgggggcgcggggctgccgcctgcgctcgcagctggtgccggtgcgcg 550

A L G L G H R S D E L V R F R F **C** B89
cgctcggcctgggccaccgctccgacgagctggtgcgtttccgcttctgc 600

S G S **C** R R A R S P H D L S L A S B106
agcggctcctgccgccgcgcgcgctctccacacgacctcagcctggccag 650

L L G A G A L R P P P G S R P V B122
cctactgggcgccgggccctgcgaccgccccgggctcccggcccgtca 700

S Q P **C C** R P T R Y **E** A V S F M D B139
gccagccctgctgccgacccacgcgctacgaagcggtctccttcatggac 750

V **N S T** W R T V D R L S A T A **C** G B156
gtcaacagcacctggagaaccgtggaccgcctctccgccaccgcctgcgg 800

C L G * B159
ctgcctgggctgagggctc 819

Figure 3